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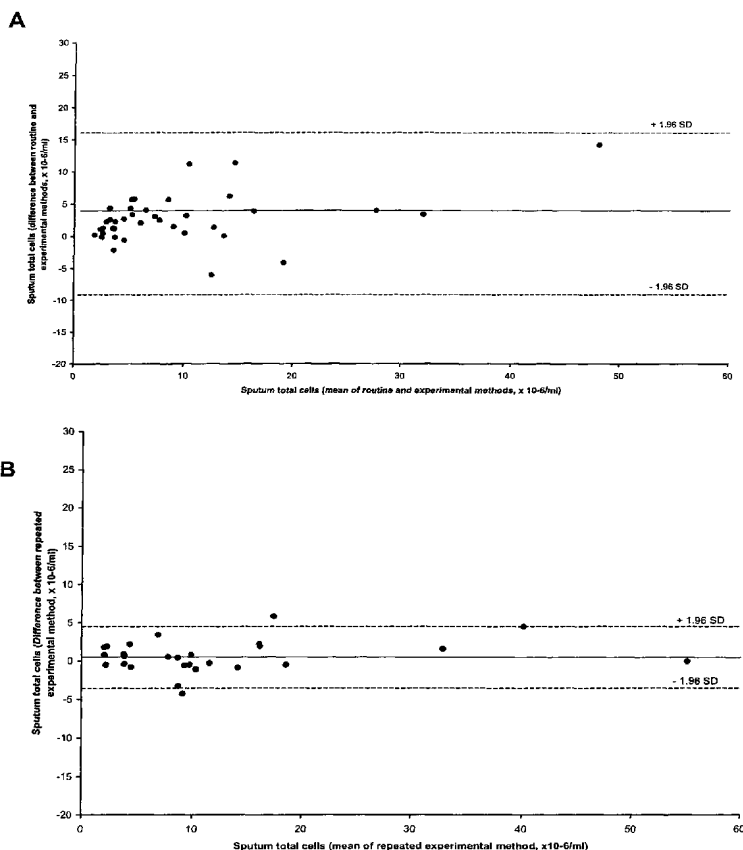
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(54) Title: SPUTUM FIXATIVE AND METHODS AND USES THEREFOR



(57) Abstract: A fixative composition for preserving cell count in a sputum sample and to a method of preserving cell count and improving cellular dispersal of the sputum sample. The composition comprises a disulphide bond reducing agent, preferably DTT, and a cross-linking agent, preferably paraformaldehyde. The method of the invention involves fixing the sputum sample and then dispersing it with a proteolytic agent such as trypsin. The method and composition are used in enhancing examination of sputum samples and can be used in the diagnosis and assessment of a number of conditions, such as airway inflammatory related conditions, i.e., asthma, chronic cough, Chronic Obstructive Pulmonary Disease, cancer, aspiration of oropharyngeal or gastric contents, including gastroesophageal reflux, heart failure, including left heart failure such as dyspnea with combined pulmonary and cardiac disorders. This invention makes possible delayed examination of the sputum sample and enables the taking of a sample at any site and time and subsequently transporting it to an appropriate centre for examination.

WO 02/44691 A2



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Title: Sputum Fixative and Methods and Uses Therefor**RELATED APPLICATION**

This application claims priority from United States Provisional Patent Application No. 60/253,121 filed November 28, 2000 entitled, "Sputum Fixative
5 and Methods and Uses Therefor", the entirety of which is incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to a sputum fixative composition and to methods and uses thereof. In another aspect, the invention relates to a method
10 of fixing and subsequent dispersal of a sputum solution, and to a method of examination of induced sputum.

BACKGROUND OF THE INVENTION

Sputum consists of a mixture of mucus, epithelial cells, inflammatory cells and cellular degradation products. In normal subjects the inflammatory
15 cells are mainly neutrophils and macrophages, with small numbers of lymphocytes, eosinophils, mast cells and basophils. Examination of the cellular morphology of sputum has prove useful in the diagnosis of conditions related to airway inflammation. It has also proven useful in establishing treatment regimes of a variety of conditions, such as asthma.

The examination of induced sputum is a minimally invasive method for investigation of airway inflammation. It can be used as a non-invasive research tool to objectively assess airway inflammation in asthma and in other conditions characterized by airway inflammation. Induced sputum cell counts have been able to detect the presence, nature, and severity of airway
20 inflammation (1,2). These studies have established induced sputum examination as a reliable and responsive non-invasive method to quantitatively assess airway inflammation in asthma. It has been suggested that the method may allow clinicians to monitor airway inflammation, and in conjunction with other indices of asthma severity, provide additional information to aid in decisions about
25 treatment requirements (2-4)/ Examination of sputum has also been found to be useful in the diagnosis of left ventricular failure (5), oesophageal reflux (6), Fabry's disease (7) and pulmonary alveolar proteinosis (8). In addition to the
30 investigation of inflammatory diseases, the use of sputum to screen for

- 2 -

malignancy has been used for several years and is an accepted technique (9).

Current methods of examination of sputum have a number limitations. Being viscid, sputum is inhomogeneous. The examination of
5 unprocessed smears has the disadvantage of sampling only a circumscribed part of the sputum which can lead to skewed examination results, when cell count, differential cell count and cellular morphology are determined. Further, the cells are entrapped in mucus and often overlap making them difficult to identify. Therefore, obtaining a well-
10 dispersed sample with release of entrapped cells and even dispersion is critical to ensure the accuracy of total and differential cell counts. Current methods use dithiothreitol (DTT) as a dispersal agent. DTT acts by reducing the disulphide bonds which cross-link glycoprotein fibres and maintain sputum in its gel form.

15 In addition to the issue of dispersal, maintaining cell count and cell morphology is also important. Since neutrophils make up a large proportion of sputum, it also contains numerous proteases (10, 11). Autolysis of sputum cells occurs after expectoration. Since accurate cell counts can only be obtained after dispersal, it is unknown whether cellular
20 autolysis occurs during dispersal itself. As cell count and quality of cellular morphology progressively declines with time, sputum currently needs to be processed (dispersed and examined) within a few hours. Therefor, a centre needs to have staff trained in the methods for handling sputum samples as well as specialised equipment. This limits the use of sputum
25 examination to larger centres which is a disadvantage for multi-centred research trials and limits its use by primary practitioners.

Therefor, there is a need for an improved method of sputum handling prior to its examination and to compositions related thereto.

SUMMARY OF THE INVENTION

30 The present inventors now provide a new sputum fixative which enhances the preservation of cell count and preferably cellular morphology and enables the delayed examination of sputum samples. In

- 3 -

another embodiment, the invention provides a method of enhanced preservation of cell count and improved subsequent dispersal of the cells. The invention provides an improved method for processing and examining sputum samples.

5 In a preferred embodiment, the invention provides a sputum fixative composition comprising a cross-linking fixative agent and a reducing agent. The cross-linking fixative agent is an agent that contains reactive aldehyde groups which cross-link proteins such as in complex chemical reactions. It is not a dehydrating fixative such as alcohol or
10 acetone. Preferably the cross-linking fixative is selected from the group consisting of paraformaldehyde, glutaraldehyde, formaldehyde and acrolein. Most preferably, the cross-linking fixative agent is paraformaldehyde.

 In another embodiment of the invention, the reducing agent is
15 an agent which facilitates subsequent dispersal of cells, preferably it reduces disulphide bonds and is preferably selected from the group consisting of DTT, DTE, and β -mercaptoethanol. Most preferably the reducing agent is DTT.

 The cross-linking fixative agent and the reducing agent are
20 present in amounts effective to preserve cell count and preferably cellular morphology of the sputum sample to enable delayed examination of the sputum.

 In the composition of the invention, the cross-linking fixative agent and reducing agent are present in a ratio of about 50:1% w/v to about
25 2:1 % w/v, but preferably about 10:1% w/v. In a preferred embodiment the cross-linking fixative agent is present in a ratio of 1:1 v/v. In a most preferred embodiment 4% w/v paraformaldehyde is added to 0.2% w/v DTT (13mM), in a ratio of 1:1, with a final concentration of paraformaldehyde of 2% w/v and of DTT of 0.1% w/v (6.5 mM).

30 In a preferred embodiment the cross-linking agent is added to the sputum sample in an amount from 1-5% w/v of the sputum sample. More preferably it is present in an amount of from about 1- 2% w/v, and

- 4 -

most preferably 2% w/v. In another embodiment, the reducing agent is added to the sputum sample in an amount from about 0.1 to 0.5 % w/v of the sputum sample. In a preferred embodiment the reducing agent is present in an amount 0.1 to 0.2% w/v, and most preferably about 0.2%
5 w/v.

In another embodiment the volume of the fixative composition of the invention, preferably paraformaldehyde DTT mixture, to that of sputum is sufficient to cover the sputum and most preferably it is at least 4 times the volume of the sputum.

10 In another embodiment the invention provides an improved method of preparing a sputum sample for examination comprising fixing the sample with the fixative composition of the invention and then dispersing the sample with a proteolytic agent, preferably selected from the group consisting of trypsin, chymotrypsin, pepsin, erepsin, bromelin, and
15 papain., most preferably trypsin. Preferably the sputum sample is fixed immediately after or upon sample collection and preferably not much greater than about 2 hours after collection, but this can vary especially depending on storage conditions of the sputum sample prior to fixation. The proteolytic agent can be added to the fixed sample from immediately
20 after fixation, up to an indefinite time period depending on when the sample is to be examined. In one embodiment the proteolytic agent is added 12 hours after addition of the fixative composition of the invention. In another embodiment it is added at about 6 months after addition of the fixative composition of the invention. Preferably it is added between
25 about 2 to 17 days after fixation, depending on when the sample is to be examined or analyzed. The proteolytic agent is added at a time prior to examination to enable sufficient dispersal of the sample for examination. Preferably it is added to allow for at least 6 hours of dispersal and most preferably for at least 6-17 hours of dispersal prior to examination of the
30 sample. The amount of trypsin used will be sufficient to reverse the fixative process, and to sufficiently disperse the cells within the sputum sample, while maintaining cell count and cellular morphology or at least

- 5 -

minimal effect thereto. Preferably it is added in an amount of between 1 to 10% w/v of fixed sample. Most preferably it is added in an amount of 2 +/- 0.5% w/v of fixed sample.

Most preferably it is added at a concentration of about 2.5% w/v
5 in a volume of about 3 times the fixed sputum by weight (i.e. 100 mg of sputum to 300 ul of proteolytic agent).

In another embodiment of the invention, the methods of the invention (including fixation of the sample, dispersal and examination of the sample are preferably carried out at between 4 to 37°C and most
10 preferably at room temperature.

In another embodiment, the invention provides a method for diagnosing and/or assessing an airway inflammatory related condition in a subject and potentially the severity thereof. Such diseases include but are not limited to asthma, chronic cough, and Chronic Obstructive Pulmonary
15 Disease, malignancy and dysplasia such as in lung cancer; in assessment of aspiration of oropharyngeal or gastric contents including in gastroesophageal reflux; and in assessment of left heart failure including dyspnea in patients with combined pulmonary and chronic cardiac disorders. In one embodiment the invention can be used to prepare a
20 sputum sample for examination by immunohistochemistry, or staining for lipid inclusions in cells, detection of malignant cells or examination for hemosiderin staining cells.

In another embodiment the invention provides a method of monitoring an airway inflammatory related condition in a subject, such as
25 asthma. In yet another embodiment the invention provides a method of determining an optimal treatment regime for a subject with an airway inflammatory related condition.

In yet another embodiment, the invention provides a kit for examination of sputum comprising the sputum fixative composition or
30 components thereof of the invention and optionally a proteolytic agent and optionally instructions for carrying out the methods of the invention.

- 6 -

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are
5 given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the
10 drawings in which:

Figure 1 shows the agreement between total sputum cell counts from aliquots from the same sample processed by the routine and experimental methods (A) and between two aliquots both processed by the experimental method (B); and

15 Figure 2 shows differential cell counts (%) of routinely and experimentally processed sputum samples.

DETAILED DESCRIPTION OF THE INVENTION

The examination of sputum has been validated as a reliable and responsive tool for the investigation of airway inflammatory disease.
20 However, sputum must be processed within a few hours, i.e. about 2 hours, requiring the presence of laboratory staff with the necessary training and equipment. To preserve sputum for later dispersal and examination, the present inventors have developed a method of fixing the sputum sample with a cross-linking agent, such as paraformaldehyde, a rapid
25 fixative with good preservation of morphology followed by proteolytic dispersion. The present inventors have also developed a novel sputum fixative composition.

The following standard abbreviations are used herein: DTT, dithiothreitol; DTE, dithioerithreitol; ICC, interclass coefficient of
30 correlation; TCC, total cell counts; IQR, interquartile range; PBS, phosphate-buffered saline; and CytoLyt, a proprietary preparation based on alcohol.

- 7 -

"Subject" as used herein is any animal of the animal kingdom that can produce sputum or a sputum like substance to be examined.

FIXATIVE COMPOSITION

The invention encompasses a sputum fixative composition which permits delayed examination of a sputum sample. The fixative composition preserves cell count and thus also improves analysis of the cellular morphology of the sample. More cells are preserved with the method and composition of the invention and eosinophils are more easily seen on differential counting.

"Sputum" as used herein refers to sputum or a sputum-like biological substance. Sputum is a mucus material produced in the airways. It is usually expectorated but also can be obtained at the time of bronchoscopy or through blind suctioning of the respiratory tract. Sputum samples in addition to mucus may contain other substances such as saliva or airway secretions.

"Biological Sample" as used herein refers to any sample comprising cells which are to be examined for cell count and/or cellular morphology, including sputum or malignant cell aggregates. Other biological samples could include cytology samples such as those used for Pap smears.

"Delayed Examination" as used herein refers to examination of the sample at some point after sample collection. This can any time after fixation of the sample. Preferably not more than 6 months after sample collection. Preferably more than six hours after sample collection, most preferably between 2 to 17 days after sample collection.

"Preserves" as used herein refers to improved maintenance of cells within the sample (i.e. reduction of cell autolysis) to enable improved examination of both total cell count and differential cell count of a sample over prior art methods.

In a preferred embodiment, the invention provides a fixative composition which can be used to fix a biological sample, preferably a

- 8 -

sputum sample comprising a cross-linking fixative agent and a reducing agent.

There are many types of fixative agents available, however, ideally, the fixative agent used in the invention should prevent autolysis of cells, preserve or provide good analysis of cellular morphology and protect the cells from osmotic damage and shrinkage during further processing.

Fixatives can be broadly grouped into 3 categories, dehydrating, precipitating and cross-linking fixatives (10). Dehydrating fixatives, such as alcohols and acetone, have a strong coagulative effect on proteins with extensive denaturation. Precipitating fixatives such as mercuric chloride, precipitate proteins by formation of insoluble salts or complexes.

The fixative agent used in the present invention is a cross-linking fixative, such as paraformaldehyde, gluteraldehyde, formaldehyde or acrolein, preferably paraformaldehyde or gluteraldehyde, and most preferably paraformaldehyde. Cross-linking fixatives contain reactive aldehyde groups which cross-link proteins, in a complex chemical reaction. Sputum, being thick and viscous, is more suitably fixed by rapidly penetrating fixatives such as those used in tissue. Paraformaldehyde at room temperature, is one of the most rapid fixatives, and provides efficient preservation without producing the shrinkage seen when alcohol based fixatives are used in tissue. Virtually no enzyme activity remains in tissue fixed in paraformaldehyde after 24 hours at room temperature. This has the advantage in sputum where the rapidly penetrating paraformaldehyde would prevent enzymic autolysis of cells. In addition, paraformaldehyde fixation has the advantage that it is partially reversible by aqueous solutions and proteolysis. Although, the fixation of the sample with paraformaldehyde at room temperature is preferable, the invention is not limited to fixation at room temperature. Fixation can be carried at a wide range of temperatures, preferably from about 4 degrees Celsius to about 37 degrees Celsius, most preferably at room temperature.

- 9 -

The process of 'antigen retrieval' has been used for several years whereby tissue fixed in paraformaldehyde is treated with combinations of various buffers, weak solutions of proteases and heat to reveal antigen moieties. This allows detection of antigens by immunohistochemistry whilst maintaining cell morphology. Treatment of paraformaldehyde-fixed, paraffin-embedded tissue by stronger protease solutions has been used to disperse tissue cells providing a single cell suspension for further analysis such as flow cytometry (11,12) and fluorescence *in situ* hybridization (13-15). The initial fixation of the tissue by paraformaldehyde allows the cells to withstand the relatively harsh subsequent treatments.

The reducing agent used in the composition is one which reduces disulphide bonds and is preferably selected from the group consisting of DTT, DTE and β -mercaptoethanol.

In another embodiment of the invention, the reducing agent is an agent which facilitates subsequent dispersal of cells, preferably it reduces disulphide bonds and is preferably selected from the group consisting of DTT, DTE, and β -mercaptoethanol. Most preferably the reducing agent is DTT.

The cross-linking fixative agent and the reducing agent are present in amounts effective to preserve cell count and preferably cellular morphology of the sputum sample to enable delayed examination of the sputum.

In the composition of the invention, the cross-linking fixative agent and reducing agent are present in a ratio of about 50:1% weight per volume (w/v) to about 2:1 % w/v, but preferably in about 10:1% w/v. In a preferred embodiment the cross-linking fixative agent is present in a ratio of 1:1 v/v. In a most preferred embodiment 4% w/v paraformaldehyde is added to 0.2% w/v DTT (13mM), in a ratio of 1:1, with a final concentration of paraformaldehyde of 2% w/v and of DTT of 0.1% w/v (6.5 mM).

- 10 -

For use, in a preferred embodiment the cross-linking agent is added to the sputum sample in an amount from 1-5% w/v of the sputum sample. More preferably it is present in an amount of from about 1- 2% w/v, and most preferably 2% w/v. In another embodiment, the reducing agent is added to the sputum sample in an amount from about 0.1 to 0.5 % w/v of the sputum sample. In a preferred embodiment the reducing agent is present in an amount 0.1 to 0.2% w/v, and most preferably about 0.2% w/v. In another embodiment the volume of the fixative composition of the invention, preferably paraformaldehyde DTT mixture, to that of sputum is sufficient to cover the sputum and most preferably it is at least 4 times the volume of the sputum.

Although, the fixative composition can be used for delayed examination of the sputum sample, preferably from about 2 days to indefinitely after sample collection and fixation of the sample a person skilled in the art would appreciate that the sputum sample can be fixed immediately or up to a few hours after collection more preferably not more than 6 hours and most preferably not more than 2 hours after sample collection. It would also be appreciated that this "fixed" sample can be immediately dispersed for immediate examination of the sample or can be dispersed at an indefinite period of time after fixation of the sputum sample (preferably at least 12 hours, more preferably at least 24 to 48 hours after addition of the fixative composition of the invention) at a time prior to examination to allow for sufficient dispersal of the sample. In one embodiment the proteolytic agent is preferably added at least 6-17 hours prior to examination.

METHODS OF THE INVENTION

In one embodiment, the invention provides a method for fixing a sputum sample and facilitating subsequent dispersal thereof comprising contacting the sample with a cross-linking fixative agent and a reducing agent which facilitates subsequent dispersal of the sample. The reducing agent preferably reduces disulphide bonds and is preferably selected from the group.

- 11 -

The cross-linking fixative agent is preferably a fast penetrating agent. It preferably has reacting aldehyde groups and is preferably selected from the group consisting of paraformaldehyde, formaldehyde, gluteraldehyde and acrolein, more preferably paraformaldehyde, 5 formaldehyde, or gluteraldehyde, and most preferably paraformaldehyde.

In one embodiment, the sample is collected from the patient, by techniques known in the art, such as asking the subject to cough or by induced expectoration (1, 11).

After sample collection, the sample is immediately fixed with 10 the cross-linking fixative agent and reducing agent as described herein, however, a person skilled in the art would appreciate that the sputum sample could be left to stand for up to about 6 hours, but preferably not more than 2 hours prior to fixation. The time can vary depending on the purpose of the examination and the type of cells which are to be detected.

15 Preferably, the cross-linking fixative agent is added to the sputum sample in an amount from 1-5% w/v of the sputum sample. More preferably it is present in an amount of from about 1- 2% w/v, and most preferably 2% w/v. In another embodiment, the reducing agent is added to the sputum sample in an amount from about 0.1 to 0.5 % w/v of 20 the sputum sample. In a preferred embodiment the reducing agent is present in an amount 0.1 to 0.2% w/v, and most preferably about 0.2% w/v. In a preferred embodiment the cross-linking fixative agent is present in a ratio of 1:1 v/v. In a most preferred embodiment 4% w/v paraformaldehyde is added to 0.2% w/v DTT (13mM), in a ratio of 1:1, with 25 a final concentration of paraformaldehyde of 2% w/v and of DTT of 0.1% w/v (6.5 mM). In another embodiment the volume of the fixative composition of the invention, preferably paraformaldehyde DTT mixture, to that of sputum is sufficient to cover the sputum and most preferably it is at least 4 times the volume of the sputum. A person skilled in the art 30 would appreciate that the fixative composition as described above can be used in the method of the invention and added to a final concentration in the ranges indicated herein.

- 12 -

The period of fixation can vary. The sputum sample can be immediately dispersed with a proteolytic agent after fixation (addition of the cross-linking fixative agent and reducing agent), however, it is unlikely that this would be preferred, as the sample at this point can be
5 adequately processed by current prior art methods. Preferably the sample is fixed for a period of from about 12 hours to indefinitely, more preferably from 12 hours to 6 months, 24 hours to 6 months or 2-17 days. More preferably the sample is fixed for two days prior to subsequent dispersal and examination. The sputum sample can be collected and fixed from a
10 temperature of about 4 to 37 degrees Celsius, and preferably at room temperature. Most preferably the sputum sample is collected and fixed at room temperature at which point it may be kept until dispersal. Although not necessary, preferably it is stored refrigerated, at about 4 to 8 degrees Celsius, until dispersal and examination of the sample.

15 Prior to examination, the sample is dispersed with a proteolytic agent, such as trypsin, chymotrypsin, pepsin, erepsin, bromelin, and papain, preferably trypsin. The amount of proteolytic agent used will be sufficient to reverse the fixative process, and to sufficiently disperse the cells within the sputum sample, while maintaining cell count and
20 preferably cellular morphology or at least minimal effect thereto. Preferably it is added in an amount of between 1 to 10% w/v of fixed sample, and more preferably in an amount from 1 to 7.5% w/v. Most preferably it is added in an amount of 2 +/-0.5% w/v of fixed sample.

As stated above, the proteolytic agent can be added to the fixed
25 sample from immediately after fixation to up to the time prior to when examination of the sample is desired to allow for sufficient dispersal prior to examination. Preferably the sample is examined within 2 to 17 days after fixation, depending on when the sample is to be examined or analyzed. The proteolytic agent is added prior to examination and
30 preferably at least 17 hours and most preferably at least 6 hours prior to examination of the sample.

- 13 -

Dispersion of the fixed sputum sample can be carried out at between 4 to 37 degrees Celsius, and preferably at room temperature when using trypsin. The conditions may vary depending on the proteolytic agent used.

5 In one embodiment, if trypsin is used it is preferably in a buffer which maintains a physiological pH, i.e. pH of 7.3-7.5 and osmolarity, i.e. 300-330. A person skilled in the art would appreciate that other conditions would work and optimal conditions may vary depending on the components used.

10 After dispersal, the sample is examined for total cell count, and where warranted, differential cell count by known methods in the art. (1) For instance total cell count in a Neubauer cell Chamber and differential cell counts on a cytopsin are standard methods of cell counting. Differential cell count is obtained by detecting the cellular morphology of
15 stained cells in the sample and comparing the abundance of one cell type with that of another or others, including total cell count, depending on what is desired to be observed or determined. A person skilled in the art would appreciate that this may depend on the condition that is to be diagnosed, or assessed. For instance, in the situation of infection, an
20 increased total cell count and % neutrophils may be expected. In a case of an asthma exacerbation, a normal total cell count and increased % eosinophils may be found.

 This invention facilitates that at the time of fixation the sputum sample is co-treated with an agent that facilitates subsequent
25 dispersion of cells and mucus. In addition, the dispersion of cells from mucus is accomplished by treatment with proteolytic enzymes. Thus, the previously observed problems of inadequate yield of cells from fixed samples is circumvented.

APPLICATIONS

30 The invention can be used for a number of applications. For instance, in one embodiment, the fixative composition of the invention as described herein can be used to fix sputum samples, preferably cells in

- 14 -

sputum samples to enhance or preserve cell count in the sample. Thus it can be used to reduce or inhibit cellular autolysis.

The immediate application of this application of this invention is to enable clinicians in distant centres to avail of a recently developed test of sputum cellularity. The test is useful in the assessment of [1] airway inflammation that occurs including in asthma, chronic cough or Chronic Obstructive Pulmonary Disease; [2] aspiration of oro-pharyngeal or gastric contents that occur in gastroesophageal reflux; [3] left heart failure as the cause of dyspnea in patients with combined pulmonary and cardiac disorders. This invention enables a sputum sample to be collected at any site and subsequently transport it to an appropriate centre for evaluation. In addition, samples can now be obtained at any time of the day or week and collection will be free of the current time constraint that samples must be delivered to the laboratory prior to 4:00PM on usual working days.

In the research area, this invention will allow the examination of sputum cellularity to be widely available to research sites without the current requirement that on-site staff be trained and available. This will allow recruitment of subjects to studies from a broad geographic area, thereby simplifying the logistics of performing research and making the subject base more generalizable.

The invention also provides an improved method of dispersing a sputum sample, or cells and/or cellular aggregates therein, after fixation. The method involves the use of a proteolytic agent as described herein. In one embodiment, the improved dispersal of the sample results in improved data collection pertaining to total and differential cell count and thus improved examination of the sample.

The method of the inventions can also be used for diagnosing and/or assessing an airway inflammatory related condition in a subject and potentially the severity thereof. Such diseases include but are not limited to asthma, chronic cough, and Chronic Obstructive Pulmonary Disease, malignancy and dysplasia such as in lung cancer; in assessment of aspiration of orthopharyngeal or gastric contents including in

- 15 -

gastroesophageal reflux; and in assessment of left heart failure including dyspnea in patients with combined pulmonary and chronic cardiac disorders. Other applications can include preparation of a sputum sample for examination by immunohistochemistry, or staining for lipid inclusions
5 in cells or deletion of malignant cells or examination for hemosiderin staining of cells.

In another embodiment the invention provides a method of monitoring an airway inflammatory related condition in a subject, such as asthma. In yet another embodiment the invention provides a method of
10 determining an optimal treatment regime for a subject with an airway inflammatory related condition.

KITS

The invention provides a kit for examination of sputum comprising the sputum fixative composition of the invention or
15 components thereof and optionally a proteolytic agent and optionally instructions for carrying out the methods and/or applications of the invention.

In one embodiment, the kit comprises a 4% w/v paraformaldehyde solution, a sample of DTT powder, instructions to
20 prepare the sputum fixative composition of the invention and optionally a proteolytic agent such as trypsin. In one embodiment, proteolytic agent can be in solution and stored frozen and transported or is a powder to be prepared and added to the fixed sputum sample. The components are provided in sufficient amounts to prepare the compositions and conduct
25 the methods of the invention.

EXAMPLES

Example 1

In a series of pilot studies, paraformaldehyde either alone at 4% w/v strength or diluted with an equal volume of 0.2% DTT w/v was used
30 to fix aliquots of selected sputum at room temperature. Varying concentrations of trypsin (working concentrations of 1-7.5% w/v, protease type XIV (working concentrations 1-2.3% w/v or chymotrypsin (working

- 16 -

concentration 1% w/v were applied to disperse the fixed sputum after 1-16 days. The time of incubation with the proteases was also varied, from 3 -17 hours. Some aliquots were also processed with 0.1% w/v DTT after fixation in a proprietary preparation based on alcohol, 'CytoLyt'. Using the
5 latter method there was a > 50% loss of total cells compared to fixation with paraformaldehyde, and the proportion lost increased with the cellularity of the specimen.

On the basis of these pilot studies, it was decided to use equal volumes of paraformaldehyde and DTT (working concentrations 2% and
10 0.1% respectively), to fix the sputum as the loss of cells was least with this method as compared to previous ones described. The fixed sputum was subsequently dispersed with trypsin (working concentration 2%), incubated at 37°C for 6-16 hours. Although the period of fixation did not appear to be critical, it was decided to allow the sputum to fix for 48-72
15 hours before dispersion to ensure uniformity. Subsequent staining of the prepared cytopins was by the manual Wright's stain, which was found to provide superior morphology for the fixed samples than the automated Wright's stain, which was used for the routinely processed samples. The manual Wright's method was also superior to staining with haematoxylin
20 and eosin, Giemsa and Diff-quick.

Example 2

The purpose of this prospective cross-sectional study was to evaluate a method whereby sputum was initially fixed for 48-72 hours before being dispersed, and the total and differential cell counts compared
25 to those obtained by the standard (or prior art) method of sputum processing. In addition, to examine the repeatability of the experimental method, a second aliquot of sputum was also processed in parallel by the experimental method.

Methods

30 **Subjects**

Sputum samples from 39 patients attending the Firestone Regional Chest and Allergy Unit at St. Joseph's Hospital, Hamilton, were

- 17 -

examined (Table 1). Sputum was produced spontaneously or after induction. 35 patients had asthma, two had chronic cough, one had chronic obstructive airway disease and one had cystic fibrosis. The diagnosis of asthma was based on the American Thoracic Society (ATS) criteria (16), and included variable airflow limitation and airway hyperresponsiveness to methacholine challenge. Based on percent predicted FEV1 (17), 16 patients with asthma had no airflow limitation (percent predicted FEV1 > 80%), 14 had mild airflow limitation, (percent predicted FEV1 60-80%), 3 had moderate airflow limitation (percent predicted FEV1 40-59%) and 2 had severe airflow limitation (percent predicted FEV1 < 40%). The patients with chronic obstructive airway disease had an onset of respiratory symptoms at > 40 years of age, > 15 pack-year smoking history, an FEV1 < 70% predicted and an improvement in FEV1 < 10% after inhaled salbutamol (16). The patients with chronic cough had a daily cough for at least one year with no evidence of asthma or other known cause for the cough. The diagnosis of cystic fibrosis was based on clinical details, positive sweat testing and genetic analysis. The study was approved by the St. Joseph's Hospital Research Ethics Committee.

Sputum induction

If sputum was not readily obtained by spontaneous expectoration, it was induced as described by Pin et al (18) and modified by Pizzichini et al (1). Briefly, after pretreatment with inhaled salbutamol, an aerosol of hypertonic saline was inhaled from a Medix ultrasonic nebulizer (Clement Clarke, Harlow Essex, UK) with a relatively low output (0.87 mL.min⁻¹) and a large particle size (5.58 mm aerodynamic mass, median diameter). Concentrations of 3, 4 and 5% v/v saline were each inhaled for 7-minute intervals. At the end of each inhalation, subjects were asked to blow their nose, rinse their mouth with water and swallow to minimize contamination with post-nasal drip and saliva. They were then asked to cough and expectorate into a polystyrene container.

- 18 -

Sputum processing

Sputum was selected from the expectorate as described by Pizzichini et al (1) within 2 hours of collection, and weighed. The selected sputum was then divided into 2-3 approximately equal aliquots (depending on the volume available), and then randomly assigned to be processed in the routine method (one aliquot) or the experimental method (one or two aliquots).

Routine sputum processing

This followed the method of Pizzichini et al (1), and consisted of the addition of 4 volumes of freshly prepared 0.1% w/v DTT (Sputolysin, Calbiochem, La Jolla, California, USA). The sputum mixture was placed on a bench rocker for 15 minutes after which 4 volumes of phosphate-buffered saline (PBS, Gibco) were added. After filtering the suspension through a 48 mm nylon mesh, the total cell count and viability were measured by trypan blue exclusion in a Neubauer haemocytometer. The filtrate was then adjusted to one million cells/ml and cytopins made (Shandon III cytocentrifuge, Shandon Southern Instruments, Sewickley, PA, USA). After air-drying, the cytopins were stained with an automated Wright's stain. A differential cell count was performed by counting 400 nonsquamous cells.

Sputum processing with the experimental method

After the sputum had been expectorated and after a variable delay (up to 2 hours), the sputum was selected as described herein and divided into aliquots. The aliquot(s) randomly assigned to this method weighed 100-400 mg, and were immediately immersed in 10 mls of a mixture consisting of 2% w/v paraformaldehyde (ICN, Montreal, Canada) and 0.1% DTT. The DTT was freshly prepared as a 0.2% w/v solution by the addition of distilled water. 4% w/v paraformaldehyde (buffered with sodium phosphate salts, pH 7.4) had been prepared within the last 4 weeks and an equal volume was added to the 0.2% w/v DTT solution

- 19 -

immediately before addition of the sputum. In 27 cases, two aliquots were available for processing by the experimental method. The sputum in the preservative solution was briefly vortexed and shaken to ensure that it sunk to the bottom of the container, otherwise it tended to float on the surface. The sputum was allowed to fix for 48 - 72 hours, after which it was centrifuged at 300g for 10 minutes. This had the effect of pelleting most of the fixed sputum and allowing most of the fixative solution to be removed with a transfer pipette. Care was taken not to remove pieces of sputum floating freely. The tube was then filled with PBS and re-centrifuged. The process of centrifugation, removal of solution, and washing with PBS was performed 3 times altogether so as to remove as much fixative solution as possible. After the third centrifugal step, the excess fluid was removed and the tube reweighed. Since the sputum container had been weighed whilst empty, the weight of sputum plus a variable but small amount of remaining fluid could be calculated. A solution of 2.5% w/v trypsin (Sigma, Mississauga, Canada) made up in 0.05M tris-buffered saline containing 0.1% w/v calcium chloride, was added to the sputum in a volume 3 times the sputum weight so that the calculated working concentration of the trypsin was approximately 2% w/v. The solution was vortexed and then incubated at 37°C for 6-17 hours. The dispersed sputum was then briefly vortexed, filtered, and the total cell count determined as in the routine method. The viability could not be estimated since many cells treated with proteolytic enzymes do not stain with trypan blue, (even they are dead) and non-viable cells are usually detected by staining with trypan-blue. In most cases, the total cell counts were performed by the same person who had done the routine total cell count. Cytospins were prepared and stained manually with Wright's stain. The slides were then coded and differential cell counts performed on 400 nonsquamous cells, by one technologist. Because it was noted in some cases that epithelial cells were numerous, in contrast to the samples handled by the usual method, where epithelial cells were usually <1%, epithelial cells were not included in the differential cell counts, but were counted separately and reported as

- 20 -

a % of the total cells. The technologist examined the slides blind to the counts obtained with the routine method.

Statistical analysis

SPSS statistical software (SPSS for Windows, Rel. 10.0.0. 1999. Chicago: SPSS Inc) was used to analyse data. Clinical data that were normally distributed were expressed as mean values with standard deviation (SD). Sputum cell counts were not normally distributed and were expressed as median values and interquartile range (IQR). Comparisons between cell counts were made by the Wilcoxon Signed Ranks test for paired data. Reproducibility was expressed by intraclass correlation coefficients as the ratio of variance among subjects to total variance. Two-tailed probability (p) values < 0.05 were considered significant.

Results

Of 39 cytospins prepared using the routine method, 5 were described as being moderately degenerate (degenerate cell changes present but 400 nonsquamous cells could still be counted), and 11 described as showing severe degeneration (numerous unclassifiable cells with poor morphology present, only 100-200 cells able to be counted). Eight cytospins prepared using the experimental method were described as 'moderately degenerate' and 6 as 'severely degenerate' which is a subjective method of assessing the quality of the cell morphology. The presence of numerous cells with dark chromatin, pyknotic nuclei, and disrupted cytoplasm would render that cytospin being described as severely degenerate. The degree of degeneration did not correlate between the two methods. One cytospin prepared using the routine method was described as unsuitable for cell counts due to poor morphology, and one cytospin prepared using the experimental method (from a different subject) was too sparse to perform cell counts. A prominent feature of the cytospins prepared using the experimental method was the presence of background deposit of varying quantity, which was seen in 7 cases. Although this deposit did not interfere with cell classification, it made counting more difficult and the

- 21 -

procedure took longer as a result. When comparing the overall staining of the cells processed by both methods, the nuclei of cells in the experimental method tended to stain more darkly. The intensity of staining of the eosinophil granules in the experimental method was often more prominent than in the routine method, so that eosinophils were easily discerned.

The median and IQR of cell counts using the routine and experimental methods are compared in Table 2. The total cell counts obtained by the routine and experimental methods were compared in all 39 subjects, with median (IQR) of $4.8 \times 10^6/\text{ml}$ (7.2) in the routine and $8.4 \times 10^6/\text{ml}$ (9.4) in the experimental method ($p < 0.001$). The interclass coefficient of correlation (ICC) for total cell counts in the routine and experimental methods was 0.89. The ICC for total cell counts obtained by processing two aliquots by the experimental method (a measure of the within-subject repeatability of the method, $n = 27$), was 0.99, with no significant difference between the total cell counts. In 36 subjects the routine and experimental differential counts were compared, with the median (IQR) of percentage eosinophils being 2.3 (8.4) and 3.5 (14.5) respectively ($p = 0.009$). The corresponding percentages of neutrophils and macrophages were 32.4 (42.2), 43.7 (56.3) ($p=0.002$) and 56.5 (48.5), 42.5 (51.9) ($p < 0.001$), respectively. The ICC for the percentage eosinophils, neutrophils and macrophages between the two methods was 0.85, 0.92 and 0.91, respectively.

Discussion

Although the ICC between the cells counts in the two methods were good, non-parametric paired tests showed that they were still significantly different. Because the total cell count was increased in the experimental method, it suggests that cells which would normally have disintegrated during routine processing (non-viable, already degenerating cells) were preserved by fixation and survived intact on the cytopins. The significantly increased median % eosinophils in the experimental method

- 22 -

may relate to better preservation of eosinophils and/or to easier detection of eosinophils which were easily identified by their intensely staining granules even when the cell cytoplasm was disrupted.

The increased median % neutrophils (and correspondingly
5 decreased % macrophages) could be explained by the fixation method inhibiting autolytic enzymes that otherwise would result in the neutrophils self-destructing in a hostile environment. The increased total cell count in the experimental method, whilst showing very good correlation with that in the routine method, is likely to be valid since the
10 within-subject repeatability of the method was excellent (ICC = 0.99) and there was no significant difference by non-parametric testing. There are two mechanisms that might explain this increase in total cell count. One is that the fixation process prevents complete disintegration of cells that would otherwise disappear during processing, such as neutrophils, which
15 autolyse, and epithelial cells, which are already non-viable and degenerate. The increased proportion of neutrophils and epithelial cells present supports this theory. Another explanation is that more efficient dispersal is achieved by trypsinisation as opposed to the use of DTT. The fixation of the cells is complementary to this in that it allows the cells to withstand
20 the relatively harsh dispersal method by proteolysis. Improved dispersion is supported by the observation of none or very small clumps of mucous being present after incubation with trypsin, in contrast to the usual method in which occasional mucous clumps are often visible. Both of these factors may be operating to increase the total cell count with the
25 experimental method.

The morphology of the cells obtained by the experimental method, although allowing differential counting, was generally not as crisp and easy to discern as that by the routine method. In the experimental method, degenerate cells appeared to be more numerous and
30 were also more prominent, as their cytoplasm stained dark blue. Degenerate cells in the routine method stained weakly and were less noticeable. The validity of the differential cell counts will be examined by

- 23 -

comparing the differential counts of the second aliquot processed by the experimental method to that of the first.

The efficient preservation of cells and their dispersal by this new method as well as being used to assess airway inflammation, could be
5 utilized in the detection of malignant cells from the airway. Sputum samples from subjects with lung cancer often contain necrotic debris, and some sort of dispersal method is recommended. However, malignant cells are more fragile and susceptible to disruption during processing than are benign cells. Presumably, many of these malignant cells do not survive
10 dispersal when applied as the initial step. The fixation step in this method would allow them to withstand processing and prevent their loss. Since they are often a small percentage of the cells present in the sample, this is important. Cells shed from a particular type of lung cancer, small cell carcinoma are especially fragile and are characteristically described in
15 sputum cytology as appearing as purple staining 'smears' of DNA which is all that is left of the cells. The method of the present invention could potentially allow more of these cells to survive and increased the diagnostic rate.

The need to select sputum is not necessary initially, and the
20 patient could cough directly into a container made up with the fixative solution. At a convenient time the sputum could then be selected after fixation, and a technologist would therefore not be required initially.

The present inventor have described a method that allows efficient, convenient preservation and dispersion of sputum, allowing its
25 examination after transportation to a tertiary specialized centre from a primary care setting. The total cell count and differential cell counts showed good correlation with the routine method which presently requires on-site technologists and specialised equipment. This method will facilitate multi-centre research and allow more widespread use of sputum
30 as a research and clinical diagnostic tool, i.e., such as lung cancer.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be

- 24 -

understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims. For instance, although, the invention is described with
5 reference to fixing, dispersal and examination of sputum, a person skilled in the art would appreciate that compositions, and methods of the invention as described herein could also be used to fix and disperse and/or examine other biological samples which have properties similar to sputum, such as potentially for fixing samples collected for pap smears. It
10 can also be used for examination of samples by immunohistochemistry or staining for lipid inclusions in cells or detection of malignant cells or examination for hemosiderin staining of cells.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each
15 individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

- 25 -

Table 1

Subject characteristics

| | |
|--|-------------|
| n | 39 |
| Gender (male) | 16 |
| Age, y (mean, SD) | 46.3 (17.3) |
| Asthma | 35 |
| Atopic (n) | 25 |
| %predicted FEV ₁ , mean (SD) | 74.1 (23.6) |
| On inhaled steroid only (n) | 18 |
| On oral prednisone and inhaled steroid (n) | 8 |

SD: standard deviation

- 26 -

Table 2

Comparison of cellular indices in sputum processed routinely and with experimental method

| | | Routine method | Exp method | Exp method (second aliquot) | ICC | p Value | | |
|--|--|----------------|-------------|--------------------------------|--------------|---------|--------------|---------|
| | | | | | Routine /Exp | Exp/Exp | Routine /Exp | Exp/Exp |
| Total Cell Count (x 10 ⁶ /g) | | 4.8 (7.2) | 8.4 (9.4) | 9.6 (11.0) | 0.89 | 0.99 | < 0.001 | > 0.05 |
| Cell Viability (%) | | 62.6 (21.0) | | | | | | |
| Eosinophils (%) | | 2.3 (8.4) | 3.5 (14.5) | | 0.85 | | 0.009 | |
| Neutrophils (%) | | 32.4 (42.2) | 43.7 (56.3) | | 0.92 | | 0.002 | |
| Macrophages (%) | | 56.5 (48.5) | 42.5 (51.9) | | 0.91 | | <0.001 | |

Exp: Experimental method

5 ICC: Intraclass correlation

IQR: interquartile range

- 27 -

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

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DETAILED FIGURE LEGENDS

Figure 1. The agreement between total sputum cell count from aliquots from the same sample processed by the routine and experimental methods (A) and between two aliquots both processed by the experimental method (B) is demonstrated by a plot of differences against mean values of total cell counts. The solid line represents the mean difference, the broken lines indicate ± 1.96 SD from the mean i.e. the 95% limits of agreement.

Figure 2. Differential cell counts (%) of routinely and experimentally processed sputum samples. The agreement between samples processed in two different ways is demonstrated by a plot of differences against mean values for eosinophils (A), neutrophils (B) and macrophages (C). The solid line represents the mean difference, the broken lines indicate ± 1.96 SD from the mean i.e. the 95% limits of agreement.

- 30 -

What is claimed is:

1. A sputum fixative composition comprising a cross-linking fixative agent and a reducing agent.
2. The sputum fixative composition of claim 1 wherein the cross-
5 linking fixative agent is selected from the group consisting of paraformaldehye, formaldehyde, gluteraldehyde and acrolein.
3. The sputum fixative composition of claim 2 wherein the cross-linking fixative agent is paraformaldehyde.
4. The sputum composition of claim 1 wherein the reducing
10 agent reduces disulfide bonds.
5. The sputum fixative composition of claim 4, wherein the reducing agent is selected from the group consisting of DTT, DTE and β -mercaptoethanol.
6. The sputum fixative composition of claim 5 wherein the
15 reducing agent is DTT.
7. The sputum fixative composition of claim 1 wherein the cross-linking fixative agent is paraformaldehyde and the reducing agent is DTT.
8. The sputum fixative composition of any one of claims 1 to 7
wherein the cross-linking agent and the reducing agent are present in a
20 ratio of 50:1 to 2:1 by percent weight per volume of the total composition.
9. The composition of any one of claim 1 to 7 wherein the cross-linking agent and the reducing agent are present in a ratio of 10:1 by percent weight per volume of the total composition.

- 31 -

10. A use of the composition of claim 8 in fixing a sputum sample wherein the composition is added to the sputum sample in an amount sufficient to fix the cells in the sputum sample and to facilitate subsequent dispersal thereof.
- 5 11. The use of claim 10 wherein the composition is added to the sputum sample to provide a final amount of:
- (a) cross-linking agent of 1 to 5% w/v of the sputum sample; and
- (b) reducing agent of 0.1 to 0.5% w/v of the sputum sample.
- 10 12. A method for preserving total cell count of a sputum sample comprising:
- (a) obtaining a sputum sample; and
- (b) adding from 1 to 5% w/v of a cross-linking fixative agent and 0.1 to 0.5% w/v of a reducing agent thereto.
- 15
13. The method of claim 12 wherein the cross-linking fixative agent and reducing agent are added in a ratio of 10:1% w/v.
14. A method of preserving total cell count of a sputum sample by adding a sputum fixative composition of any one of claims 1 to 11 to the
- 20 sputum.
15. The method of claims 12 to 14 wherein the cross-linking fixative agent and the reducing agent are added to the sample prior to significant cellular autolysis and degradation.
16. The method of claim 15 wherein the cross-linking fixative
- 25 agent and reducing agent are added immediately after to about 2 hours after sample collection.

- 32 -

17. The method of any one of claims 12 to 14 wherein the sample is fixed at between 4 to 37°C.
18. The method of claim 17 wherein the sample is fixed at room temperature.
- 5 19. A method for preserving and dispersing a sputum sample comprising:
- (a) obtaining a sputum sample;
 - (b) fixing the cells in the sample by adding a cross-linking fixative agent and a reducing agent to the sample in an amount sufficient to preserve cell count and facilitate subsequent dispersal of the sample; and
 - (c) dispersing the cells in the sample by adding an effective amount of a proteolytic agent wherein the amount of proteolytic agent used does not damage the cells of the sputum sample.
- 10
- 15
20. The method claim 19 wherein the cross-linking fixative agent and reducing agent are added to the sputum sample obtain a final concentration of 1 to 5% w/v of cross-linking agent and 0.1 to 0.5% w/v of reducing agent.
- 20 21. The method claim 20 wherein a compositions as described in any one of claims 1 to 9 is used.
22. The method claim 19, wherein the proteolytic agent is selected from the group consisting of trypsin, chymotrypsin, pepsin, erepsin, bromelin and papain.
- 25 23. The method of claim 22, wherein the proteolytic agent is trypsin.

- 33 -

24. The method claim 22, wherein the proteolytic agent is added to the fixed sample in an amount of from 1 to 10 % w/v of fixed sample.
25. The method claim 24 wherein the amount of proteolytic agent added to the fixed sputum sample is $2 \pm 0.5\%$ w/v of the fixed sample.
- 5 26. The method of claim 19 wherein the proteolytic agent is added 2 to 17 days after fixation.
27. The method of claim 19 for examining total and/or differential cell count of sputum sample wherein the proteolytic agent is added at least 17 hours prior to examination.
- 10 28. The method of claim 27 wherein the proteolytic agent is added at least 6 hours prior to examination.
29. The method of claim 19 carried out at between 4° to 37°C .
30. The method of claim 29 carried out at room temperature.
31. The method of any one of claim 19 to 31 for diagnosing and/or
15 assessing an airway inflammatory related condition.
32. The method of claim 31 wherein the airway inflammatory condition is selected from the group consisting of: asthma, chronic cough, Chronic Obstructive Pulmonary Disease, malignancy, dysplasia, lung cancer, aspiration of orthopharyngeal or gastric contents,
20 gastroesophageal reflux and left heart failure.
33. The method of claim 32 wherein the left heart failure is dyspnea combined with the pulmonary and chronic cardiac disorders.

- 34 -

34. A kit for carrying out any one of the methods of claims 12 to 33 comprising a composition of any one of claims 1 to 9 or components thereof

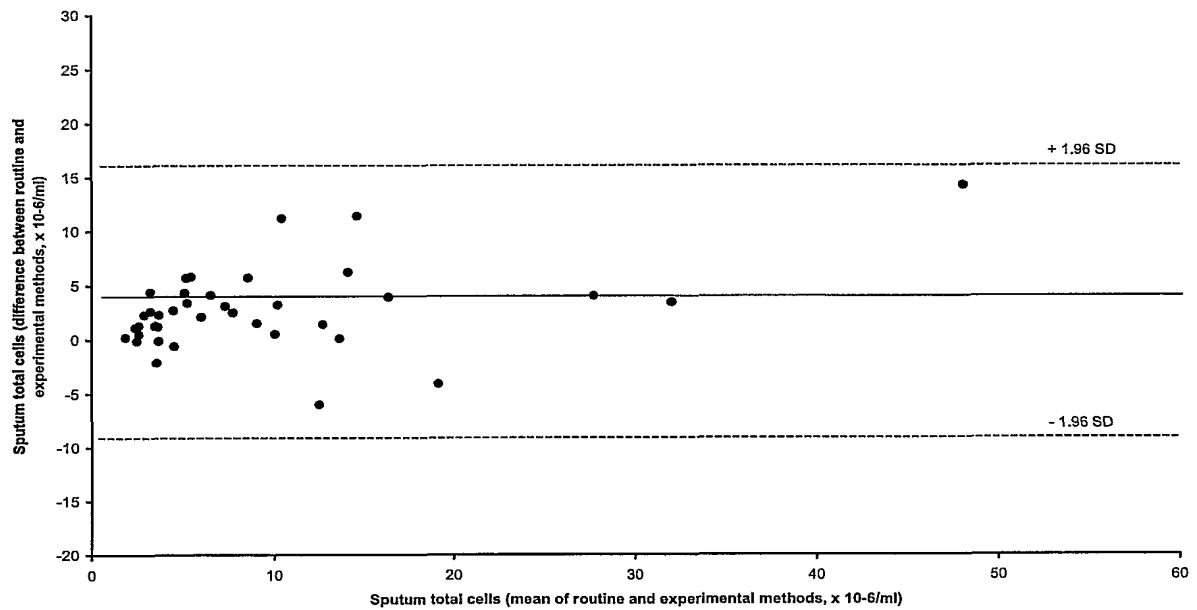
35. The kit of claim 34 further comprising a proteolytic agent.

5 36. The kit of any one of claims 34 and 35 also comprising instructions for carrying out the methods of the invention.

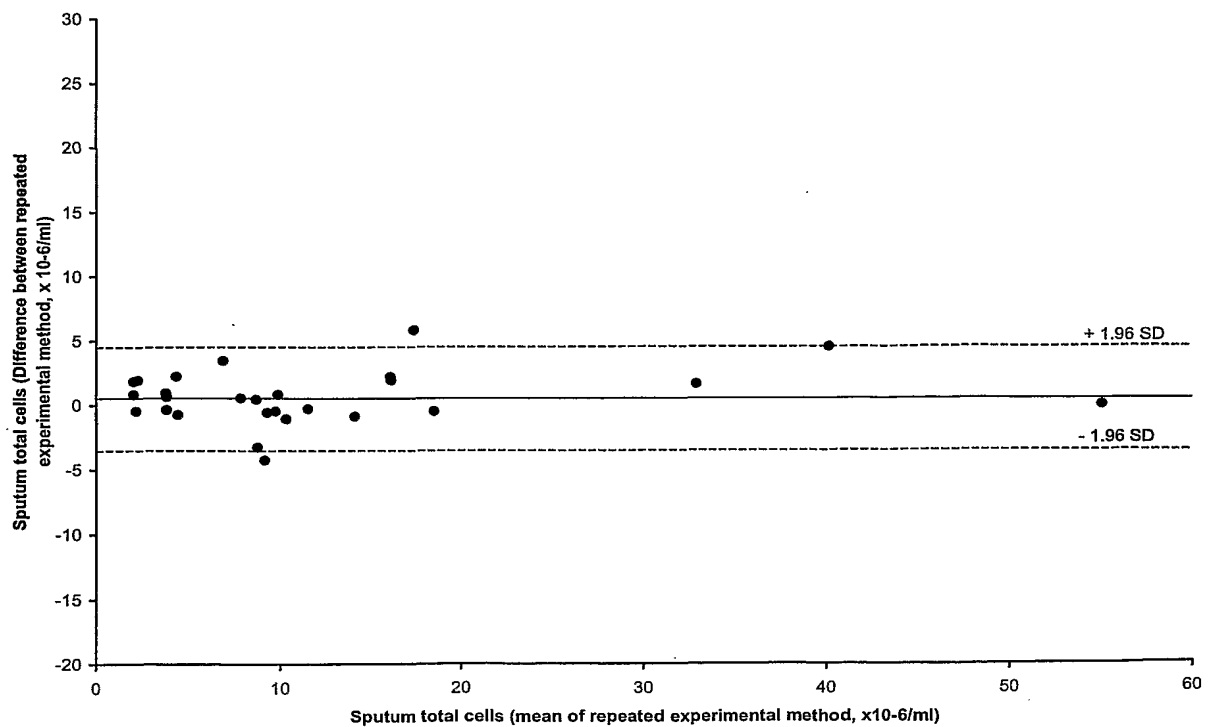
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FIGURE 1

A



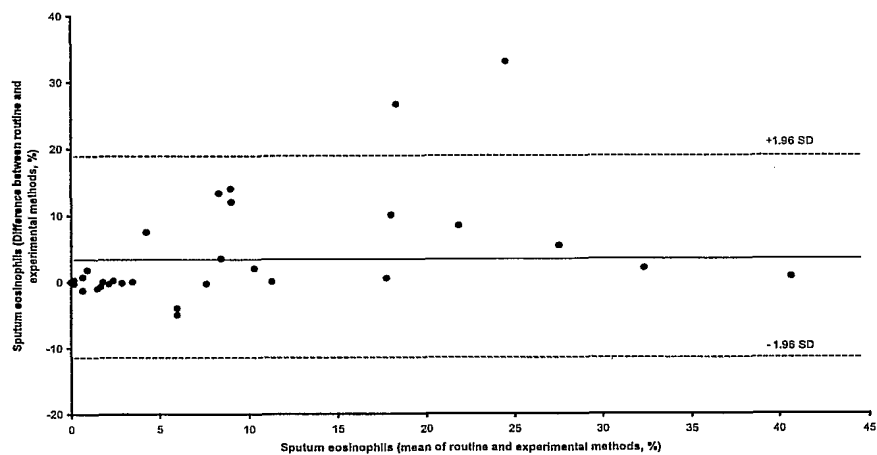
B



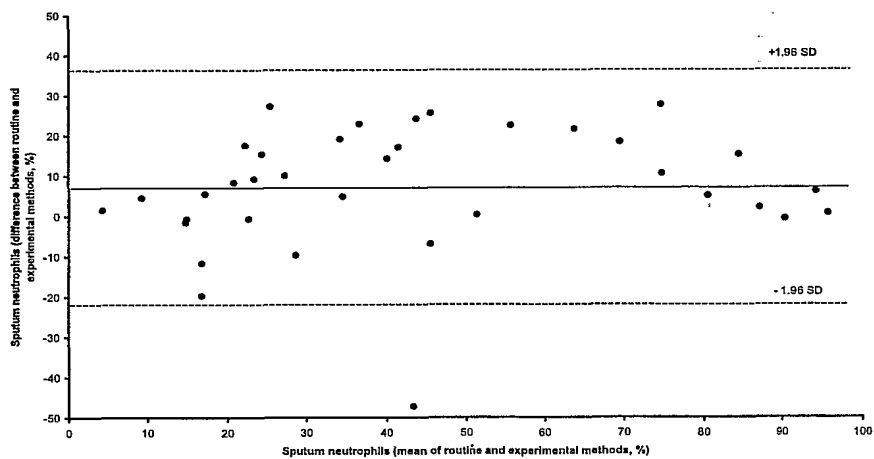
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A

FIGURE 2



B



C

